

H //

REVIEW OF THE 1972-1976 LITERATURE ON THE HEALTH ASPECTS
OF COPPER SALTS AS FOOD INGREDIENTS

1977

Prepared for

Bureau of Foods
Food and Drug Administration
Department of Health, Education, and Welfare
Washington, D. C.

Contract No. FDA 223-75-2004

by

Michael J. Wade, Ph.D.

Life Sciences Research Office
Federation of American Societies
for Experimental Biology
9650 Rockville Pike
Bethesda, Maryland 20014

FOREWORD

The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB) provides scientific assessments of topics in the biomedical sciences. Reports are based upon comprehensive literature reviews and the scientific opinions of knowledgeable investigators engaged in work in specific areas of biology and medicine.

This technical report was prepared for the LSRO Select Committee on GRAS Substances (SCOGS) as a part of their review of the health aspects of using these food ingredients as stipulated in the Food, Drug, and Cosmetic Act for Generally Recognized as Safe substances. Dr. Michael J. Wade prepared the report based on a comprehensive search and evaluative assessment of the current literature in accordance with the provisions of contract no. FDA 223-75-2004. Acknowledgment is made of the assistance of the LSRO staff who provided much of the background information.

The report was reviewed and approved by the LSRO Advisory Committee (which consists of representatives of each constituent society of FASEB) under authority delegated by the Executive Committee of the Federation Board. Upon completion of these review procedures, the report was approved and transmitted to FDA by the Executive Director, FASEB.

While this is a report of the Federation of American Societies for Experimental Biology, it does not necessarily reflect the opinion of all of the individual members of its constituent societies.

C. Jelleff Carr, Ph.D.
Director
Life Sciences Research Office

TABLE OF CONTENTS

	Page
Foreword	iii
Introduction	1
I. Acute Toxicity of Copper Salts	3
II. Short-term Studies	5
III. Special Studies	11
IV. References Cited	21

INTRODUCTION

This report concerns the health aspects of using copper salts as food additives. It reviews the world's scientific literature from 1972 through 1976.

To assure completeness and currency as of the date of this report, information has been obtained by searches of new, relevant books, abstract journals, and reviews and the literature citations contained in them; consideration of current literature citations obtained through computer retrieval systems of the National Library of Medicine, recent literature searches by the Toxicology Information Response Center, Oak Ridge, Tennessee, and by the combined knowledge and experience of members of the LSRO staff. This report supplements and updates information contained in a scientific literature review (monograph) prepared for FDA by Informatics, Inc.¹ which summarizes the world's scientific literature up to 1974.

¹The document is available from the National Technical Information Service, U.S. Department of Commerce, P.O. Box 1553, Springfield, Virginia 22161.

I. ACUTE TOXICITY OF COPPER SALTS

There are three recent reports of acute copper toxicity in humans involving ingestion of from 1 to 50 g of copper sulfate. Severe intravascular hemolysis and methemoglobinemia developed in a 27-year-old male who was admitted to the hospital 2 hours after ingesting 50 g of copper sulfate (770 mg copper sulfate per kg body weight) (Chugh *et al.*, 1975). Peak blood copper concentration was 8,267 μg per 100 ml (normal 214 ± 52 μg per 100 ml). Despite peritoneal dialysis and intravenous fluid therapy, the patient developed severe hemolysis, cyanosis and oliguria 5 hours after ingestion of the compound. He was further treated with ascorbate and methylene blue but suffered circulatory collapse, hypotension, shock and coma and died 16 hours following the ingestion of copper sulfate.

Another fatal case of copper poisoning occurred when it was used as an emetic to treat a case of alcohol-diazepam intoxication (Stein *et al.*, 1976). When 10 ml of a 10 percent copper sulfate solution (15.4 mg copper sulfate per kg body weight) failed to elicit vomiting in the 44-year-old female patient, her stomach was lavaged with 2 liters of saline in an attempt to remove the copper. Nevertheless, symptoms of copper sulfate toxicity developed including hemolytic anemia, hemoglobinuria and hepatic and renal failure. Massive gastrointestinal bleeding and death occurred on the 6th day after ingestion of the copper sulfate. At autopsy there was acute renal tubular necrosis and the hepatic copper level was 7.46 mg per 100 g of tissue (normal 0.8 mg per 100 g). A medical history revealed that 3 years previously, the patient had a 3/4 gastrectomy which may have affected the copper absorption.

Hemodialysis was found to be ineffective in treating a 41-year-old woman with vomiting and diarrhea. She was hospitalized 1 hour after drinking 1/2 pint of copper sulfate solution (concentration not reported) (Agarwal *et al.*, 1975). Immediate treatment consisted of gastric lavage and 1 g of ethylenediamine tetracetic acid given intravenously. Twelve hours following admission, hemodialysis was administered for 5 hours; however, it was not effective in lowering blood copper levels which were 2.05 and 1.95 mg percent after 2 and 5 hours of dialysis respectively. The patient subsequently suffered hemolysis, and hepatic and renal failure and died on the 6th day of hospitalization.

II. SHORT-TERM STUDIES

There are numerous reports in the literature concerning effects of feeding of copper salts to animals. Sensitivity to copper salts varies greatly in domesticated farm animals; sheep may become fatally poisoned by consuming a diet containing as little as 27 ppm copper (1.08 mg per kg body weight per day), whereas ponies have thrived and reproduced normally on a diet supplying 791 ppm copper (15.8 mg per kg body weight per day). One-day-old Hubbard and Leghorn chicks were fed a diet containing 1,000 ppm added copper, as copper sulfate (125 mg Cu per kg body weight per day). After 14 days these animals showed 46 percent less weight gain and 40 percent reduced feed consumption as compared to animals fed a basal diet containing only 55 ppm added copper. There was no mortality at the 1,000 ppm feeding level (Jensen, 1975).

Smith *et al.* (1975) fed yearling Shetland and Shetland-Welsh ponies a basal diet which contained 8 ppm total copper (0.32 mg copper per kg per day). The diet of the ponies was then supplemented with cupric carbonate to supply 262, 458, or 719 ppm copper (5.24, 9.16, and 15.6 mg Cu per kg body weight). The latter value represents an intake of about 2 g of copper daily. After 6 months on these diets none of the animals had any visible signs of toxicity. There was a statistically insignificant trend of lower weight gain with increasing dose of copper carbonate. Whole blood and plasma copper levels did not increase with increasing time or dosage of added dietary copper. Plasma enzyme level of glutamic oxaloacetic transaminase and lactic dehydrogenase taken after 136 and 183 days of copper feeding showed no significant increases over the control animals receiving 8 ppm copper in the basal diet. There was a significant decrease in both activities after 183 days of feeding at the highest copper level. (Sheep exhibit increased serum glutamic oxaloacetic transaminase and lactic dehydrogenase activities several weeks prior to undergoing a hemolytic crisis due to copper feeding.) After 84 days of copper feeding a liver biopsy was performed on one pony from each copper feeding level. On a dry weight basis, liver copper values increased from 20 ppm in the pony fed the basal diet to 1,325 ppm in the pony fed 15.6 mg cupric carbonate per kg per day in its diet. After 183 days of copper feeding, biopsies of liver, kidney, and muscle were taken from two ponies fed the 15.6 mg per kg per day cupric carbonate diet. The two ponies showed liver copper levels of 4,295 and 3,445 ppm. Based on reported normal values in sheep and cattle, the kidney copper levels of 125 and 94 ppm may have been slightly elevated. Muscle copper values of 6.6 and 3.0 ppm fall within the range found in cows.¹ Urine samples taken from all the

¹Smith *et al.* (1975) found no data available for normal copper levels in pony muscle.

ponies at 170 days of the trial showed no urinary copper, based on a minimal detectable level of 0.02 ppm. Between three and four months after termination of the trial, five of the ponies fed increased copper dropped normal healthy foals.

Sheep are very susceptible to chronic copper poisoning. Typically two phases are involved, first a period of tissue accumulation of copper with minimal toxic signs followed by a hemolytic phase with pathological changes in the liver, kidney, and other organs. Gopinath *et al.* (1974) conducted a study of the effect of copper intake on the kidney and liver of sheep. Sixteen animals were given a daily drench of aqueous cupric sulfate corresponding to 20 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per kg body weight (5.05 mg copper per kg body weight per day). These animals and nine controls were fed a diet containing about 7 ppm copper (0.28 mg Cu per kg body weight per day). Animals were sacrificed at 3, 5, 7 and 9 weeks after commencement of copper dosing. The remaining test animals were allowed to develop hemolysis and were sacrificed or died at various stages of the hemolytic crisis. There was no evidence of gross pathological changes occurring in the livers or kidneys of test animals prior to the onset of hemolysis. Upon histological examination there was an increase of eosinophilic, intracytoplasmic granules in the epithelium of the proximal convoluted tubules of the treated sheep. By contrast, kidneys of animals killed during the hemolytic phase were markedly changed. They were swollen and dark brown or black colored with little demarcation between the cortex and medulla. Pinpoint black foci were seen on the surface and fine black streaks were present on transverse sections of the renal cortex. The cells lining the proximal convoluted tubules of sheep in hemolytic crisis showed increased eosinophilic granules and abundant cytoplasmic granules. Most granules were periodic acid-Schiff positive and stained for iron, copper, and hemoglobin. Two animals were killed in the posthemolytic phase. The kidneys were blue-black and swollen. Vacuolation, focal necrosis, presence of intracytoplasmic bodies and desquamation of tubular epithelium most markedly in proximal convoluted tubule cells were seen upon histological examination. Swelling was noticed in cells of the distal convoluted tubules. Eosinophilic granular or hyaline casts were frequent in both the cortex and medulla, many stained for hemoglobin. A patchy increase in fibroblast activity and early collagen deposition in the interstitial tissue of the cortex were noted. Livers of animals killed during the hemolytic phase were friable and pale or yellow. Blood stained pericardial fluid and epicardial hemorrhage were noticed in some animals. Animals killed after hemolysis showed less marked jaundice but had pale soft livers. Histochemical studies showed no enzyme activity loss in kidney sections taken from seven animals in the pre-hemolytic phase when stained for glutamic dehydrogenase, alkaline phosphatase, or glucose-6-phosphatase. There was a slight increase in acid phosphatase activity in sections from three of seven of the sheep; however, sheep in the hemolytic and posthemolytic phases showed loss of glutamic dehydrogenase, alkaline phosphatase and glucose-6-phosphate dehydrogenase activities in

cells of the proximal convoluted tubules. Kidney samples taken during early hemolysis showed a marked increase in acid phosphatase-positive granules in the epithelial cell cytoplasm. Up to the time of hemolysis, urinary values for pH, glutamic-oxaloacetate transaminase activity, protein concentration and creatinine showed no significant changes. At hemolysis the urine was coffee colored, contained abundant amorphous granular material, showed increased protein concentration and glutamic oxaloacetate transaminase levels. There was also a drop in the creatinine clearance rate, and the urinary pH changed from alkaline to acid. Serum copper levels increased two- to fivefold over the prehemolytic values at the onset of hemolysis. Mean copper, iron and zinc values in ppm (dry weight) for liver and kidney at different phases of copper toxicity are shown in Table I.

Gopinath and McC. Howel (1975) studied the changes in serum enzyme and urea levels in eight sheep given daily doses of 30 mg per kg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (7.6 mg Cu per kg) in 0.5 percent aqueous solution. These animals and the controls were fed a basal diet containing about 7 ppm copper (0.28 mg per kg per day). Hemolytic crises occurred in the copper dosed animals from 5 weeks to 5 months after the onset of dosing. In the prehemolytic phase there were small gradual rises in serum sorbitol dehydrogenase, glutamic dehydrogenase and glutamic oxaloacetate transaminase. There were large increases in the serum enzyme levels (more than tenfold in the animal for which data were supplied). Four sheep died or were killed *in extremis* during their first hemolytic crisis. In the four sheep surviving the first hemolytic crisis, serum enzyme levels returned to normal at the cessation of hemolysis. Three of the animals developed additional hemolytic crises and the serum enzyme levels again rose. Prior to and during these crises, all animals that died or were killed *in extremis* during hemolytic crises showed a seven- to sixteenfold increase in serum urea over prehemolytic values, and were jaundiced with yellow-brown discoloration on the carcass. The livers of these animals were soft and yellow and histological examination showed the majority of the liver cells in the mid- and peripheral zones of the hepatic lobules were enlarged and vacuolated; some cells showed karyomegaly. Foci of necrotic cells and varying degrees of inflammatory cell infiltration were scattered throughout the lobules. Yellow pigment was deposited in the cytoplasm of many cells and in the canaliculi. Kidneys taken from animals killed or dying *in extremis* during the first hemolytic crisis were swollen and black. The proximal tubules showed epithelial necrosis, desquamation, and vacuolation. Many surviving proximal tubule cells were eosinophilic. Granular casts were found in cortical and medullary tubules. Livers and kidneys of two sheep who survived the hemolytic crises showed less marked gross lesions; the livers appeared pale and the kidneys were slightly swollen and dark brown. However, these animals also exhibited some histological changes such as enlargement of periportal and midzonal liver cells. Isolated necrotic cells surrounded by polymorphonuclear leucocytes were scattered through the lobules. In the kidney, eosinophilic

TABLE I

Metal Concentration in Kidney and Liver^a During Different Phases of
Copper Toxicity

	Control ^b	Prehemolytic ^c	Hemolytic ^c	Posthemolytic ^c
Liver copper	208.6±36.1	2150.8±179.0	2442.0±285.4	4251.6±922.3
Kidney copper	15.6± 0.1	46.6± 6.7	547.4±108.9	410.5± 89.9
Liver iron	141.73±87.9	----	172.8± 77.5	148.3± 70.9
Kidney iron	101.2±17.0	101.2± 17.0	213.3± 46.1	981.5±165.1
Liver zinc	63.2±12.7	63.6± 9.8	81.2± 10.9	147.4± 42.1
Kidney zinc	94.4± 3.7	94.4± 3.7	141.6± 14.2	139.7± 23.8

^a Values in ppm (dry weight basis)

^b Basal diets fed both control and experimental animals provided about 0.28 mg copper per kg body weight per day

^c Received 5.05 mg copper per kg body weight per day as CuSO₄·5H₂O

Adapted from Gopinath et al. (1974).

granules were noticed in the epithelial cells of the proximal tubule; dilatation of many distal tubules and foci of interstitial connective tissue proliferation were found in the cortex.

Tait *et al.* (1971) reported on the accidental poisoning of feeder lambs fed rations which included a dairy cattle mineral ration with added copper sulfate. Total copper in the diet of the lambs was 27 ppm (1.08 mg Cu per kg per day). Thirteen of fifty-five lambs died about 16 to 18 weeks after being fed the copper supplemented rations and an additional six lambs were killed because icterus was evident throughout the carcass. The lungs of the poisoned sheep were "liver-like" in appearance with signs of severe hemorrhaging. The liver was dull yellow and the kidneys tended to be enlarged and dark brown in color. Hemosiderin laden macrophages were observed in the alveolar lung tissue. Fatty degeneration of the hepatic cells was noted with an increased number of hemosiderin laden reticulo-endothelial cells. Nephrosis and tubular occlusion with hemoglobin casts were observed in the kidney sections. Liver samples taken at time of slaughter ranged from 1,017 to 1,538 ppm copper on a dry weight basis, compared to normal values for sheep of 100 to 400 ppm. Serum copper values of jaundiced lambs averaged 2.5 ppm compared to normal values of 0.6 to 1.5 ppm.

Howel *et al.* (1974) described changes in the central nervous system of sheep administered a daily oral dose of 0.5 percent copper sulfate, corresponding to 5.05 to 7.58 mg copper per kg body weight. The 29 copper-dosed and 13 control sheep were fed a diet containing 7 ppm copper (0.28 mg per kg). At various times, 23 of the sheep were anesthetized and their brains fixed by perfusion via the carotid artery with a 10 percent formulin solution containing 1 percent acetic acid or 4 percent glutaraldehyde in phosphate buffer. In 11 other animals killed by exsanguination and eight animals that died, the brains and spinal cords were taken out as quickly as possible after death and fixed in neutral formulin. No changes were seen in brains from control sheep or from copper treated sheep prior to the onset of hemolytic crisis, which occurred from 6 to 27 weeks subsequent to the beginning of copper dosing. Status spongiosis was seen in five of eleven animals that died or were killed during hemolysis, but it was extensive in only two animals. It was present in seven of nine animals that died or were killed in the posthemolytic phase; however, the lesion was extensive in four of the seven. Six animals had multiple hemolytic episodes; five of those exhibited status spongiosis; in two the lesion was extensive. The status spongiosis involved white matter in the brain and spinal cord. Multiple vacuoles were often seen around single nerve fibers. Changes were noted in the astrocytes of hemolytic and post-hemolytic animals, and the nuclear volumes of astrocytes from tissue fixed by immersion were significantly larger in tissues of hemolytic and post-hemolytic animals. Examination by light and electron microscopy showed no abnormalities in neurons of any of the sheep.

Morgan (1973) also reported on the changes in the brains of sheep poisoned with copper. Five lambs, age 6 months, were given a drench of 1 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (5.6 mg per kg) five times a week. These animals and three controls were fed a diet containing 5.5 ppm copper (0.22 mg per kg per day). Of the five copper-treated lambs, one died of unstated causes on day 15 of the experiment. The other four underwent hemolytic crises from 4 to 10 weeks after the onset of dosing and were killed *in extremis*. The four copper-treated animals and the three controls were anesthetized and their brains perfused via the common carotid artery with a mixture of 1 percent glutaraldehyde and 4 percent formaldehyde in phosphate buffer. The brains were removed and sections prepared for light and electron microscopy. The central nervous system showed extensive areas of spongiform leucoencephalopathy, which were confined to the brain stem, cerebellum and spinal cord. The lesion was characterized by clear spaces around large myelinated axons. Apart from compression by adjacent spongy tissue, neurons, glia and blood vessels were unaffected. Electron microscopic examination showed the clear spaces around myelinated axons to be vacuoles produced by separation of lamellae at the intraperiod line. Mitochondrial and glycogen granules present in the vacuoles appeared to derive from nearby ruptured astrocyte processes. Oligodendroglia and neurons were apparently normal. Histological examination of the liver revealed extensive necrosis and loss of centrilobular hepatocytes with karyomegaly and nuclear vacuolation in many liver cells. There was also infiltration of polymorphonuclear leucocytes and large quantities of bile pigment were observed in bile ducts and canaliculi.

Kojima and Tanaka (1973) reported on the toxicity of copper sulfate when administered to male dd mice. Groups of animals were given from 1.6 to 0.006 percent copper sulfate (407 - 1.52 mg per kg body weight per day) in their drinking water, in a decreasing progression with each concentration of CuSO_4 being one-half the preceding one. The experiment lasted 15 days. Four of the five animals given 1.6 percent copper sulfate in their water died before the 15th day. Animals given 0.4 percent (102 mg Cu per kg per day) or higher CuSO_4 levels showed markedly slower growth than controls given tap water to drink. Animals given 0.8 percent (204 mg Cu per kg) and 1.6 percent CuSO_4 lost weight during the experiment, while the controls and those receiving 0.2 percent (51 mg Cu per kg) or less CuSO_4 had about a 40 to 50 percent weight gain. The copper content was 4 μg per g wet weight in the liver of control rats and was not markedly elevated at the lower copper feeding levels. Animals receiving 0.2 percent CuSO_4 had a liver copper content of 16.3 μg per g. Animals receiving 0.8 percent copper had 178.4 μg per g copper in their liver.

III. SPECIAL STUDIES

A. TERATOGENICITY OF COPPER SALTS

Both copper sulfate and copper citrate when injected intravenously into 8-day-pregnant hamsters caused a marked increase in both embryonic resorptions and developmental abnormalities (Ferm and Hanlon, 1974). Copper citrate was more toxic to the mothers and caused more resorptions and abnormalities than an equivalent dose of copper sulfate. Copper sulfate was teratogenic in the range of 2 - 10 mg copper injected per kg body weight, while copper citrate was teratogenic in the range of 0.25 - 4.0 mg copper injected per kg body weight, the higher dose being maternicidal in each case. Developmental abnormalities noted included thoracic wall hernia, eucephalocoeles, spina bifida and tail defects (the latter seen only with copper citrate treatment). The authors speculated that copper citrate was more teratogenic because the free copper ion in the copper sulfate solution would be immediately bound to serum albumin and transported to the liver, whereas the copper citrate chelate may be more directly carried to maternal and fetal tissues where it produces its toxic effect.

B. CARCINOGENICITY OF COPPER SALTS

As discussed in the monograph¹, and recently reviewed by Schwartz (1975), elevations in tissue copper levels have been found in patients with a variety of neoplastic diseases and in some metabolic disorders. Another recent review by Clayson (1975) mentioned no publications citing increased carcinogenesis by feeding of copper salts, and cited several studies showing that feeding copper salts had no effect on, or in some cases inhibited tumors induced by chemical carcinogens.

Furst (1971) found no lymphomas or fibrosarcomas among a group of weanling rats injected monthly in the thigh muscle with 45 mg (450 mg per kg body weight) of metallic copper powder suspended in an oil vehicle. The experiment was continued for 450 days. When mice were injected under similar conditions with nickel, 76 percent of them developed fibrosarcomas.

¹ Monograph on copper salts, TR-72-1552-52 submitted to the FDA by Informatics, Inc., 6000 Executive Boulevard, Rockville, Maryland 20852.

The metabolism of the carcinogen 4-dimethylaminoazobenzene (DAB) was altered in the livers of rats fed copper salts (Yamane and Sakai, 1974). Rats were fed diets supplemented with varying amounts of copper acetate, copper sulfate or magnesium, nickel or zinc acetate. After 2 weeks, it was found the activity for azo reduction of DAB was doubled in livers of rats fed 0.5 percent (171 mg Cu per kg per day) $\text{Cu}(\text{C}_2\text{H}_5\text{O}_2)_2 \cdot \text{CuO} \cdot 6\text{H}_2\text{O}$ (cupric oxyacetate) as compared to control animals fed no supplemental minerals. DAB ring hydroxylation activity was only enhanced 1.2-fold and N-demethylation of DAB was decreased about 40 percent in the livers of rats fed the copper-supplemented diet. The liver copper content of the rats fed copper $(\text{C}_2\text{H}_5\text{O}_2)_2 \cdot 6\text{H}_2\text{O}$ was 115 μg per g of liver¹ compared to 4.4 μg per g in control animals. Compared to control animals there was a 1.6-fold increase in azo reduction and a 36 percent reduction in demethylation activities in the livers of rats fed a diet supplemented with 0.5 percent $\text{Mn}(\text{C}_2\text{H}_5\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ (181 mg Mn per kg body weight). Only small changes in the metabolism of DAB occurred in the rats fed 0.5 percent zinc acetate or 0.1 percent nickel acetate (29.9 mg Ni per kg body weight).

The effect of feeding 0.5 percent $\text{Cu}(\text{C}_2\text{H}_5\text{O}_2)_2 \cdot \text{CuO} \cdot 6\text{H}_2\text{O}$ on liver DAB metabolism was compared to the effect of feeding 0.06 percent (60 mg per kg body weight per day) phenobarbitol (PB) or 3-methyl-cholanthrene (3-MC); the latter two are known inducers of liver drug metabolizing enzymes. After one week on the diets, azo reduction was increased about twofold over control values in rats fed the copper, PB or 3-MC diets. Ring hydroxylation was increased about fourfold in the PB fed rats and only 1.3- and 1.5- fold in the rats fed 3-MC or copper acetate. Copper feeding caused a 45 percent drop in liver N-demethylation of DAB whereas it increased 70 percent in rats fed 3-MC or PB. Experiments with isolated microsomes showed the increases in the liver activities for azo reduction and ring hydroxylation of DAB due to copper feeding were mainly localized in the microsomes. Copper content of the microsomal fraction from the rats fed 0.5 percent $\text{Cu}(\text{C}_2\text{H}_5\text{O}_2)_2 \cdot \text{CuO} \cdot 6\text{H}_2\text{O}$ for one week was 1.55 μg per g compared to 0.6 μg per g in control animals. Rats fed diets containing 0.25 percent (85.1 mg Cu per kg per day) or 0.1 percent (35.2 mg Cu per kg per day) $\text{Cu}(\text{C}_2\text{H}_5\text{O}_2)_2 \cdot \text{CuO} \cdot 6\text{H}_2\text{O}$ did not show significant increases in liver DAB azo reduction or ring hydroxylation activities. Liver copper content was also not increased at these feeding levels. Rats fed 0.5 percent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (127 mg Cu per kg body weight per day) in their diets showed changes in liver DAB metabolism similar to those seen with rats fed 0.5 percent $\text{Cu}(\text{C}_2\text{H}_5\text{O}_2)_2 \cdot \text{CuO} \cdot 6\text{H}_2\text{O}$. In rats fed 0.5 percent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, liver copper content did increase significantly to 0.92 μg per g compared to 0.54 μg per g in control animals.

¹ Authors did not state whether wet weight or dry weight basis.

C. MUTAGENICITY

Cupric chloride was found not to be mutagenic to *B. subtilis* based on the "rec-assay" which measures the differential growth sensitivities to potential mutagens in wild type and repair deficient cells (Nishioka, 1975). No other studies concerning the possible mutagenic effects of copper salts were found for the time period 1972-1976.

D. INTERACTIONS OF COPPER WITH OTHER TRACE ELEMENTS IN THE DIET

There are a number of reports concerning the interactions of copper with other trace elements in the diet.

1. Copper and Molybdenum Interactions

Seelig (1973) synthesized data from a number of sources in support of her thesis that there is a high copper/molybdenum ratio in the American diet which may play a role in iron deficiency and iron storage diseases. Seelig cited published examples of correlation between high serum copper and low serum iron in pregnant women and other studies showing a better hemoglobin response in anemic pregnant women when they were given molybdenum in conjunction with ferrous sulfate than when they were given ferrous sulfate alone. Although she proposed that the elevated copper levels of pregnant women may lead to a conditioned molybdenum deficiency, no direct evidence of an actual antagonism between the two elements was presented. She also postulated that the high copper/molybdenum ratio of fetal liver may reflect a mechanism that inhibits the mobilization of iron from fetal liver and may be associated with anemias of premature and newborn infants. Seelig also cited studies showing high plasma levels of copper in anemias associated with rheumatoid arthritis and bacterial infection and other studies showing low molybdenum levels in iron deficiency, nutritional, hemorrhagic, and neoplastic associated anemias. There are reports of a copper/molybdenum antagonism in ruminants.

Huisingh *et al.* (1973) reviewed the three-way interaction of copper, molybdenum and sulfate which occurs in ruminant nutrition. High dietary molybdenum produces symptoms of copper deficiency which can be reversed by supplemental copper feeding. In ruminants, high levels of sulfate also cause copper deficiency symptoms which can be reversed by copper feeding. This is evidently due to a sulfate reductase system in the rumen which causes copper to form an insoluble precipitate with the newly formed sulfide. Molybdenum feeding can reverse the copper insufficiency brought about by high sulfide levels. Huisingh *et al.* (1973) proposed that molybdate may inhibit

the sulfate reductase system by competing with a sulfate carrier protein. Sulfate can alleviate symptoms of molybdenum-induced copper deficiency, perhaps by inhibiting molybdenum resorption in the kidney distal tubules. This inhibition could be mediated by competition for binding to a hypothetical molybdenum carrier protein in the distal tubules.

Although dietary molybdenum can apparently induce copper deficiency in ruminants, feeding studies in sheep have shown that increased dietary molybdenum can cause increased plasma copper levels. Smith and Wright (1975a) studied the effect of molybdenum on the binding of copper to plasma proteins in sheep. They fed sheep a diet which provided 10 mg of copper (0.16 mg Cu per kg per day) and 0.2 mg of molybdenum daily (0.0033 mg molybdenum per kg). Groups of sheep received molybdenum supplementation of 0, 8, 16, or 24 mg of molybdenum per kg diet (0, 0.32, 0.64, 0.96 mg molybdenum per kg body weight per day) (chemical form of molybdenum not stated). The experiment lasted 203 days; all changes noted took place within 12 days and were reversed 12 days after ending molybdenum supplementation. Sheep given 16 and 24 mg per kg diet (high molybdenum diets) showed a significant increase of about 50 percent in total plasma copper over the animals given 0 or 8 mg per kg diet additional molybdenum (low molybdenum diets). The increase could not be accounted for by an increase in serum ceruloplasmin which did not markedly increase in the high molybdenum diet. The values for trichloroacetic acid (TCA) insoluble serum copper were about fiftyfold greater in sheep on the high molybdenum diets than in animals on the low molybdenum diets. The authors speculated that increased dietary molybdenum causes increased binding of copper to a serum protein(s) in such a way that the copper is not removed by TCA treatment. In another report the same authors found a similar copper-molybdenum interaction in guinea pigs (Smith and Wright, 1975b). When the diet of guinea pigs (containing 6 mg Cu per kg of diet) (0.24 mg Cu per kg body weight per day) was supplemented with 100 µg per g molybdenum as ammonium molybdate (4.0 mg molybdenum per kg body weight per day), there was an increase in total plasma copper from 820 to 3220 µg per liter. Most of the increase was accounted for in TCA-insoluble copper which went from the 30 µg per liter seen in controls, to 1980 µg per liter in the molybdenum-fed animals. The addition of molybdenum to the diet of the guinea pig did not significantly affect liver copper levels.

2. Iron and Copper Interactions

Copper levels up to 250 ppm have been fed to swine and poultry as a purported growth stimulant. The interrelationship of dietary iron and copper at high copper feeding levels, was studied by Hedges and Korngay (1973). Sixty young pigs, average weight 8.9 kg, were allotted to six dietary schemes for 9 weeks. A factorial 2 x 3 design was used with two levels of iron feeding, and three levels of copper feeding. Supplemental iron and copper were added as the sulfate salts. Total copper levels were 7, 25, or 257 ppm (0.3, 1.0, or 10.3 mg Cu per kg per day), and total iron levels were 101 or 312

ppm (4.0 and 12.5 mg per kg per day). The feeding of 257 ppm copper significantly increased weight gain only during the first 4 weeks of the trial, but with no increase in the feed/gain ratio. Hemoglobin levels were significantly reduced at weeks 3, 6, and 9 in pigs fed 257 ppm copper and 101 ppm iron; however, the effect of 257 ppm copper on hemoglobin levels was reversed when 312 ppm iron was fed. The authors speculated that the high copper level may inhibit absorption of iron from the gut, possibly by competing with iron for a binding site. Ceruloplasmin levels were significantly reduced at both iron feeding levels after 9 weeks feeding with 257 ppm copper. Serum iron concentration was significantly reduced in pigs fed the diet containing 257 ppm copper and 101 ppm iron. Liver copper values were in the range of 21-23 ppm dry weight basis, in pigs fed 7 and 25 ppm copper at both iron levels. However, pigs fed 257 ppm copper in the diet had 505 ppm copper in the liver at an iron feeding level of 101 ppm, and 112 ppm copper in the liver at an iron feeding level of 312 ppm.

Gipp *et al.* (1974) studied the effect of iron and ascorbic acid on the anemia which occurred when 3-week-old pigs were fed a diet containing 250 ppm copper (10 mg per kg body weight per day). The pigs were fed a basal diet containing 100 ppm iron (4 mg per kg per day), 10 ppm copper (0.4 mg per kg per day) and 0.011 percent ascorbate (4.44 mg per kg per day). One group of animals received 240 ppm additional copper, as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (9.6 mg Cu per kg body weight) in the diet for 5 weeks. This group exhibited signs of iron deficiency, including significantly reduced values for hemoglobin, packed cell volume, plasma iron and percentage saturation of plasma transferrin. Another group was given 200 ppm iron, as FeSO_4 , (8 mg Fe per kg body weight per day) in addition to the 250 ppm copper. The supplemental iron feeding reduced the degree of change in hematological parameters due to copper but did not return any parameters to basal levels. Ascorbic acid at 0.5 percent (20 mg per kg body weight per day) in the diet was able to completely overcome the reductions in hemoglobin parameters caused by 250 ppm copper feeding. This level of ascorbate feeding also caused significant increases in hemoglobin parameters in pigs fed the basal diet. Liver iron values were not affected by any of the diets, but the high dietary copper caused an increase in liver copper from 34 ppm (dry weight) in the basal diet to 6,080 ppm in the high copper diet. This high value was about the same in the high copper-high iron diet and reduced to 3,335 ppm in the diet containing 250 ppm copper and 0.5 percent ascorbate. High dietary copper caused about a fourfold increase in the kidney copper levels; these were reduced below basal levels by high ascorbate, but not reduced significantly by high iron. Animals on the high copper diet showed significant reductions in the iron content of a segment of the duodenum. There was also a significant reduction of plasma zinc levels in pigs on the high copper diet. Studies using ^{59}Fe showed the pigs fed high copper diets had no build up of radioiron in the liver or kidney and a rapid clearance of an injected dose of ^{59}Fe from the plasma. A high level of the injected ^{59}Fe reappeared in the red blood cells indicating a fully functional hemoglobin-producing system. These results led the authors to speculate that the high copper feeding caused anemia by impairment of iron uptake from the gastrointestinal tract.

3. Zinc and Copper Interactions

The interrelationships between copper and zinc nutrition were studied in rats fed diets low in zinc ($0.7 \mu\text{g per g}$) ($0.07 \text{ mg per kg body weight}$) and copper ($0.4 \mu\text{g per g}$) ($0.04 \text{ mg per kg body weight}$) with additional copper and zinc given in the drinking water (Murthy *et al.*, 1974). A factorial experiment was designed with groups of rats given water containing 2.5 to $40 \mu\text{g per ml}$ zinc ($0.35 - 5.6 \text{ mg per kg body weight}$) as zinc acetate and 0.25 to $2 \mu\text{g per ml}$ ($0.035 - 0.28 \text{ mg per kg body weight}$) copper as cupric sulfate. The experiment lasted 60 days and at the feeding levels used, there was no effect of copper on growth. In heart, brain, aorta, esophagus, skin, spleen and testis tissues there were no changes in copper and zinc values at any of the feeding levels. Kidney copper levels increased with increasing doses of copper, and there was apparently no effect of dietary zinc or copper on the kidney level of the other. Increases in dietary zinc caused a decrease in liver copper, whereas dietary copper levels had no effect on zinc liver level.

Klevay (1973) found that an increase in the ratio of zinc to copper intake apparently caused hypercholesterolemia when fed to weanling rats for up to 270 days. The purified diet of the rats provided $1 \mu\text{g per g}$ copper ($0.1 \text{ mg per kg per day}$) and $2 \mu\text{g per g}$ zinc ($0.2 \text{ mg per kg per day}$), amounts insufficient to permit normal growth and hemoglobin synthesis. Graded amounts of supplementary zinc, 10 to $20 \mu\text{g per ml}$ ($1.4 - 2.8 \text{ mg per kg}$) and copper, 0.25 to $0.5 \mu\text{g per ml}$, ($0.035 - 0.070 \text{ mg per kg per day}$) were added to the distilled drinking water. The control group always had a ratio of zinc to copper in their drinking water of 5, obtained by having $10 \mu\text{g per ml}$ zinc and $2 \mu\text{g per ml}$ copper in their water. The experimental group had a ratio of zinc to copper of 40 produced by adding either 10 or 20 ppm zinc and 0.25 or 0.5 ppm copper to the water. The growth rate of the experimental group was 66 to 74 percent of that of a group of animals fed commercial laboratory chow. Plasma levels of cholesterol were increased in the experimental groups by 15 to 34 percent over control groups when the animals remained on their respective diets from 45 to 151 days.

Strain *et al.* (1975) reported on a 6-month-old male infant suffering from acrodermatitis enteropathica who also had a high ratio of serum copper to zinc. His serum copper was $140 \mu\text{g per 100 ml}$, and serum zinc $35 \mu\text{g per ml}$ compared to normal adult values of $100 \mu\text{g copper per 100 ml}$ and $125 \mu\text{g zinc per 100 ml}$. Sixteen days after beginning zinc gluconate therapy (7.5 mg zinc , 3 times daily) the patient had a serum zinc level of $130 \mu\text{g per 100 ml}$ and $125 \mu\text{g per 100 ml}$ copper. Prior to and during zinc treatment the patient was receiving di-iodohydroxyquinoline (325 mg , four times daily).

4. Copper-selenium Interactions

In a two-week feeding study Jensen (1974) found that addition of 1,000 ppm copper, as the sulfate salt ($25 \text{ mg Cu per kg body weight}$), to the

diet significantly reduced the mortality associated with feeding chicks a diet containing 40 to 80 ppm selenium (5 - 10 mg per kg). The addition of copper to the diet of the selenium-treated chicks also partly reversed the adverse effect of selenium upon weight gain and feed consumption. Feeding 1000 ppm copper increased up to fifteenfold the selenium content of the livers of chicks fed diets supplemented with 5 to 80 ppm selenium (0.63 - 10 mg per kg), but not in chicks fed the unsupplemented diet which contained about 1 ppm selenium (0.13 mg per kg).

E. CYTOTOXIC EFFECTS OF COPPER SALTS *IN VITRO*

Ward *et al.* (1975) investigated the suppressive effect of copper chloride and other metal cations on rabbit leucocyte and fibroblast function. At 3×10^{-4} M concentration CuCl_2 caused a 50 percent inhibition of leucocyte chemotaxis. Similar concentrations of ferrous, ferric and manganous chloride caused 50 percent inhibition of leucocyte chemotaxis. The cupric chloride inhibition of chemotaxis could be mostly reversed by washing the leucocytes after a 20-minute incubation in cupric chloride. Cupric chloride caused an inhibition of protein synthesis in rabbit leucocytes and fibroblasts. In the presence of 10^{-4} M CuCl_2 , leucine incorporation into leucocytes was inhibited by 33 percent, and proline incorporation into fibroblasts by 99 percent. At 10^{-4} M concentration, zinc chloride produced almost identical inhibition of protein synthesis, while ferrous chloride was almost ineffective and manganous chloride was only effective against uptake of leucine into leucocytes. Cuprous chloride at 10^{-3} M caused a cytotoxic release of 28 percent of a ^{51}Cr dose from HeLa cells, but only 12 percent release from fibroblasts. No ^{51}Cr was released from HeLa cells or fibroblasts when they were treated with 10^{-4} M CuCl_2 .

Jecht and Bernstein (1973) found that the motility of human sperm cells was inhibited upon exposure to copper sulfate. Inhibition was arbitrarily defined as reduction of sperm motility to 10 percent of that observed with sperm not exposed to copper. When the sperm were suspended in seminal plasma their motility was not inhibited by exposure to 10^{-3} M copper sulfate. Sperm washed once with Ringer's solution were inhibited by exposure to 10^{-3} M CuSO_4 and twice washed sperm were inhibited by 10^{-4} M CuSO_4 .

Naeslund (1972) found that *in vitro* incubation of mouse blastocysts with 10^{-4} M cupric chloride inhibited both zonal loss and outgrowth. Normal outgrowth and zonal loss occurred in blastocysts incubated *in vitro* with 10^{-8} M cupric chloride.

F. BIOCHEMICAL STUDIES

The sulfhydryl group content of a protein fraction from liver increased 5.6-fold over controls when mice were injected every third day with 50 μ moles per kg body weight of cupric sulfate (Nagahashi, 1974). The mice were injected a total of six times. Animals injected with 100 μ moles per kg cupric sulfate died within three days of the first injection. A tenfold increase in sulfhydryl content occurred in the same liver protein fraction when mice were injected every third day with 15 μ moles per kg of cadmium acetate. The authors speculated that the protein fraction may actually be metallothionin since it has similar properties to that protein.

Copper salts may react chemically with other substances in the diet. For example, Brada *et al.* (1975) showed that ethionine can form a complex with copper acetate. When the two substances were administered together orally, changes took place in the absorption, toxicity and metabolism of ethionine.

G. CLINICAL STUDIES

Walker-Smith and Blomfield (1973) have reported a fatal case of possible chronic copper poisoning in a 14-month-old male infant who was admitted to the hospital with ascites and elevated serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase enzyme levels. Liver biopsy showed severe micronodular cirrhosis. Increased copper in the liver nodules was detected by rubeanic acid staining. Plasma free copper level was elevated at 70 μ g per 100 ml, normal range 5 - 15 μ g per 100 ml, whereas the plasma caeruloplasm level was only 15 μ g per 100 ml, normal 20 - 52. The child's home had copper plumbing and raised copper levels in the water, 675 μ g per 100 ml copper was found when the cold water was first turned on compared to hospital water of 15 μ g per 100 ml. However, 24-hour urinary copper levels were normal in the parents and sibling. When the family members were challenged for excess copper storage by chelation with penicillamine, only the mother showed elevated urinary copper which, however, still fell inside the normal adult range. Although the child was treated with penicillamine, he still became jaundiced and died 6 weeks after presentation. At necropsy the liver copper levels were about 330 mg per 100 g dry tissue compared to a normal value of 2 mg per 100 g and values of 75 - 176 mg per 100 g in patients with Wilson's disease. Cerebral copper levels were two- to fivefold higher than normal but less than in some cases of Wilson's disease. The authors concluded that the infant either suffered

from chronic copper poisoning or from Wilson's disease, which was exacerbated by high copper in the drinking water. In relation to the other members of the patient's family, he may have been especially sensitive by being exposed to high copper levels *in utero*. The fetus may be sensitive to copper because of the occurrence of neonatal mitrochondrocuprein in fetal liver which has a tenfold greater copper binding capacity than "adult" hepatocuprein.¹ Thus the patient may have been born with a very high level of hepatic copper and have been unable to excrete it due to high copper intake.

¹This protein is described by Porter (1965).

IV. REFERENCES CITED

- Agarwal, B.N., S.H. Bray, P. Bercz, R. Poltzker and E. Labovitz. 1975. Ineffectiveness of hemodialysis in copper sulphate poisoning. *Nephron* 15: 74-77.
- Brada, Z., N. Altman and S. Bulba. 1975. The effect of cupric acetate on ethionine metabolism. *Cancer Res.* 35: 3172-3180.
- Chugh, K.S., P.C. Singhal and B.K. Sharma. 1975. Methemoglobinemia in acute copper sulfate poisoning. *Ann. Intern. Med.* 82: 226-227.
- Clayson, D.B. 1975. Nutrition and experimental carcinogenesis: a review. *Cancer Res.* 35: 3292-3300.
- Ferm, V.H. and D.P. Hanlon. 1974. Toxicity of copper salts in hamster embryonic development. *Biol. Reprod.* 11: 97-101.
- Furst, A. 1971. Trace elements related to specific chronic diseases: cancer. *Geol. Soc. Mem.* 123: 109-130.
- Gipp, W.F., W.G. Pond, F.A. Kallfelz, J.B. Tasker, D.R. Van Campen, L. Krook and W.J. Visek. 1974. Effect of dietary copper, iron and ascorbic acid levels on hematology, blood and tissue copper, iron and zinc concentrations and ^{64}Cu and ^{59}Fe metabolism in young pigs. *J. Nutr.* 104: 532-541.
- Gopinath, C., G.A. Hall and J. McC. Howell. 1974. The effect of chronic copper poisoning on the kidneys of sheep. *Res. Vet. Sci.* 16: 57-69.
- Gopinath, C. and J. McC. Howell. 1975. Experimental chronic copper toxicity in sheep. Changes that follow the cessation of dosing at the onset of haemolysis. *Res. Vet. Sci.* 19: 35-43.
- Hedges, J.D. and E.T. Kornegay. 1973. Interrelationship of dietary copper and iron as measured by blood parameters, tissue stores and feedlot performance of swine. *J. Anim. Sci.* 37: 1147-1154.
- Howell, J. McC., W.F. Blakemore, C. Gopinath, G.A. Hall and J.H. Parker. 1974. Chronic copper poisoning and changes in the central nervous system of sheep. *Acta Neuropathol.* 29: 9-24.
- Huisingh, J., G.G. Gomez and G. Matrone. 1973. Interactions of copper, molybdenum, and sulfate in remnant nutrition. *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* 32: 1921-1924.

- Jecht, E.W. and G.S. Bernstein. 1973. The influence of copper on the motility of human spermatozoa. *Contraception* 7: 381-401.
- Jensen, L.S. 1975. Modification of selenium toxicity in chicks by dietary silver and copper. *J. Nutr.* 105: 769-775.
- Klevay, L.M. 1973. Hypercholesterolemia in rats produced by an increase in the ratio of zinc to copper ingested. *Am. J. Clin. Nutr.* 26: 1060-1068.
- Kojima, R. and E. Tanaka. 1973. Effect of oral administration of copper sulphate to mice. *Exp. Anim.* 22: 247-250. (In Japanese).
- Morgan, K.T. 1973. Chronic copper toxicity of sheep: An ultrastructural study of spongiform leukoencephalopathy. *Res. Vet. Sci.* 15: 88-95.
- Murthy, J.L., L.M. Klevay and H.G. Petering. 1974. Interrelationships of zinc and copper nutriture in the rat. *J. Nutr.* 104: 1458-1465.
- Naeslund, G. 1972. Blastocystotoxic effect of copper *in vitro*. *Contraception* 6: 281-285.
- Nagahashi, M., O. Wada, T. Ono, N. Yamaguchi and K. Takeo. 1974. Dose-response relationships between given doses of heavy metals and sulfhydryl group contents in the special protein fractions extracted from tissues of mice. *Ind. Health* 12: 31-36.
- Nishioka, H. 1975. Mutagenic activities of metal compounds in bacteria. *Mutat. Res.* 31: 185-189.
- Porter, H. 1965. The tissue copper proteins: cerebrocuprein erythrocuprein, hepatocuprein and neonatal hepatitis mitochondriocuprein. *In*: J. Peisach, P. Aisen and W.E. Blumberg, eds. *The biochemistry of copper*, proceedings of the symposium of copper in biological systems. Academic Press, New York, N.Y.
- Schwartz, M.K. 1975. Role of trace elements in cancer. *Cancer Res.* 35: 3481-3487.
- Seelig, M.S. 1973. Proposed role of copper-molybdenum interaction in iron-deficiency and iron-storage diseases. *Am. J. Clin. Nutr.* 26: 657-672.
- Smith, J.D., R.M. Jordan and M.L. Nelson. 1975. Tolerance of ponies to high levels of dietary copper. *J. Anim. Sci.* 41: 1645-1649.
- Stein, R., D. Jenkins and M.F. Korn. 1976. Death after use of cupric sulfate as emetic. *J. Am. Med. Assoc.* 235: 801.

Smith, B.S.W. and H. Wright. 1975a. Copper: molybdenum interaction. Effect of dietary molybdenum on the binding of copper to plasma proteins in sheep. *J. Comp. Pathol.* 85: 299-305.

Smith, B.S.W. and H. Wright. 1975b. Effect of dietary Mo on Cu metabolism. Evidence for the involvement of Mo in abnormal binding of Cu to plasma proteins. *Clin. Chim. Acta* 62: 55-63.

Strain, W.H., F.S. Hirsh and B. Michel. 1975. Increased copper/zinc ratios in acrodermatitis enteropathica. *Lancet* 1: 1196-1197.

Tait, R.M., C.R. Krishnamurti, E.W. Gilchrist and K. MacDonald. 1971. Chronic copper poisoning in feeder lambs. *Can. Vet. J.* 12: 73-75.

Walker-Smith, J. and J. Blomfield. 1973. Wilson's disease or chronic copper poisoning? *Arch. Dis. Child.* 48: 476-479.

Ward, P.A., P. Goldschmidt and N. D. Greene. 1975. Suppressive effects of metal salts on leukocyte and fibroblastic function. *J. Reticuloendothel. Soc.* 18: 313-321.

Yamane, Y. and K. Sakai. 1974. Effect of basic cupric acetate on biochemical changes in the liver of the rat fed carcinogenic aminoazo dye. III. Effect of copper compared with some other metals, phenobarbital and 3-methylcholanthrene on the metabolism of 4-dimethylaminoazobenzene. *Chem. Pharm. Bull. (Tokyo)* 22: 1126-1132.